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Preparation of Fluorescent Microcystin Derivatives via Direct Arginine Labelling and their Biological Evaluation

Verena Grundler[a], Susanne Faltermann[b], Karl Fent[b,c] and Karl Gademann*[a]

Abstract: Microcystin constitutes the most prevalent toxin produced by cyanobacteria and poses a severe threat to livestock, humans and whole ecosystems. We report the preparation of a series of fluorescent microcystin derivatives through direct arginine labelling of the unprotected peptides at the guanidinium side chain. This new method allows for a late-stage diversification strategy of native peptides devoid of protecting groups under mild and operationally simple conditions. A series of fluorophores have been conjugated to microcystin-LR in good to very good yield. The fluorescent probes displayed comparable biological activity to unlabelled microcystin in both phosphatase inhibition assays and toxicity tests on the crustacean *Thamnocephalus platyurus*. In addition, we demonstrated that the fluorescent probes penetrated Huh7 cells. Whole animal imaging was performed on *T. platyurus*, and the labeled compound was mainly observed in the digestive tract.

Introduction

Climate change, eutrophication, and increase in agricultural and other activities have led to an exacerbation of cyanobacterial blooms in water bodies worldwide. Their impact on fisheries, tourism, recreational activity and the whole ecosystem is considered severe, as often drinking water supplies need to be monitored or beaches closed. This issue is further complicated by the fact that various toxic secondary metabolites are produced by cyanobacteria.[1-4] The release of these compounds in the context of cyanobacterial blooms poses a serious threat for water supply, public health and for livestock and wildlife worldwide.[5-7] Microcystins (MC) constitute the most abundantly-observed toxins and over 80 different congeners of these cyclic non-ribosomal depsipeptides have been described. In this domain, microcystin-LR (MC-LR) occupies a prominent position, as it constitutes the most extensively studied toxin due to its potent LD_{50} value (50 μgkg^{-1} in rats).[8] The World Health Organization (WHO) has set a maximal guideline value of 1 μgL^{-1} for MC-LR in drinking water, which further emphasizes its potency and toxicity.[9,10] In humans, this substance shows a hepatotoxic effect through inhibition of protein phosphatases 1 (PP1) and 2A (PP2A) and the chronic exposure to this toxin can cause liver cancer.[11-13] MC-LR also causes a disruption of the cytoskeletal components accompanied by cell deformation initiated by cytokeratin hyperphosphorylation, which ends in a collapse of the whole liver architecture.[14] Moreover, MC-LR has been reported to cause DNA-damage, apoptosis, disruption in the cell-signalling and endoplasmic reticulum stress.[15-17] The known transport of MCs in the cell is mediated by the organic anion polypeptide transporter (OATP), *e.g.* in human hepatocytes.[18,19] In aquatic organisms, which are particularly vulnerable to cyanobacterial microcystins due to the increased levels of exposure, such toxic effects are also displayed. For example, in *Daphnia magna*, which is an important grazer of cyanobacteria as a food source, disturbance in the reproduction together with higher mortality rates and deteriorated development are observed.[20-24] In fish and invertebrates, the toxin accumulates in the tissues and leads to a number of toxic effects.[25,26]

Even though microcystins have been known for decades, the reasons for the strong toxicity in animals are not yet fully understood. To gain an insight into the uptake, distribution, accumulation and excretion of MC-LR, biological studies using chemical microcystin probes are necessary. In this study, we have prepared fluorescently labelled MC-LR in order to monitor its behaviour, uptake and excretion in aquatic animals.

First, a site suitable for chemical modification that did not impact the biological activity of MC had to be identified. It has been reported that the reactive Mdha groups bind in a Michael-type addition covalently to Cys 273 of the protein phosphatase 1 and the Adda side chain interacts with the hydrophobic cage of the active pocket.[27] Furthermore, inspection of the crystal structure of the MC-LR/PP1 complex shows that leucine and the carboxylate groups are also involved in enzyme binding.[28] Interestingly, this crystal structure reveals that the arginine side chain protrudes from the binding pocket suggesting that the Arg residue is not participating in the inhibition of the phosphatase and, thus, presents a promising site for modification. However, efficient acetylation of the arginine guanidine group proved to be difficult due to the high pK_a value of this protonated side chain (pK_a = 12.5). Shreder and co-workers have successfully introduced a fluorophore at this moiety in MC-LR with a compromised yield by using a two-step procedure.[29] Similar yields were obtained by a comparable reaction to a MC-LR-DNA conjugate.[30] In consideration of the limited availability of the toxin, improved methods are needed to modify MC-LR. We

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recently reported a method for the late-stage modification of the arginine side chain in unprotected, native peptides.[31]
In this study, we will present (1) the application of this method in the context of labelling microcystin with a number of fluorophores; (2) biological evaluation of the labelled compounds in a series of enzymatic, cellular, and toxicity assays in animals, and (3) a distribution study of the labelled microcystin derivatives in the ecotoxicological relevant crustacean *Thamnocephalus platyurus*.

Scheme 1. Fluorescently labeled MC-LR derivatives via direct arginine modification

Results and Discussion

As already discussed, modification of the arginine residue in microcystin-LR should not interfere with enzyme binding, as suggested by inspection of the crystal structure of the microcystin/phosphatase complex.[28] Furthermore, hypervariations in the amino acid sequence at this position in natural occurring microcystins support the hypothesis that this residue is central for the inhibitory effect. To find an effective labelling method for MC-LR, we had to consider several difficulties: (a) The poor reactivity of the guanidinium group, (b) MC-LR contains other reactive groups like the reactive unsaturated Mdha residue, (c) MC-LR gets degraded under strong basic or acidic conditions, and (d) the availability of the toxin, which can only be isolated in small amounts.[32]
Thus, different strategies for labelling this moiety had to be evaluated, and based on our recently published method, [31] we opted for the use of Barton's base. The coupling of MC-LR was performed under standard conditions by dissolving the toxin in DMF and adding Barton's base. After initial stirring at 40 °C and subsequent addition of the different, dissolved activated ester, the reaction was continued for eight hours. To our delight, the desired products functionalized with different fluorescent reported groups were obtained in moderate to good yields (55 % to 84 %). Utilizing this method, four fluorescently labelled derivatives were synthesized (**Scheme 1**).

After successfully labelling MC-LR with different fluorophores, we investigated their photochemical properties. The maximum absorption of the derivatives was at 499 nm for MC-LR-(5,6-FAM), at 425 nm for MC-LR-(Alexa-430) and at 586 for MC-LR-(Texas-Red) (see supporting information). Furthermore, the maximum emission is located at 525 nm for MC-LR-(5,6-FAM), at 434 nm for MC-LR-(Alexa-430) and at 605 nm for MC-LR-(Texas-Red). With this data, we were able to select the right compounds for the various uptake studies, with regard to the overlap of the intrinsic fluorescence of the used compounds and biological test systems. To visualize the spectral colour of the different compounds, colour space chromaticity diagrams were recorded. MC-LR-(5,6-FAM) is located more in the yellowish-green range (x: 0.32 y: 0.66), whereas MC-LR-(Alexa-430) is already in the yellow-green area (x: 0.38 y: 0.58) and MC-LR-(Texas-Red) is situated in the orange-yellow section (x: 0.65 y: 0.36).

Figure 1. Phosphatase inhibition assay **A:** with MC-LR-(5,6-FAM) IC_{50} = 0.37 nM **B:** with MC-LR-(Alexa-430) IC_{50} = 0.61 nM

With the desired derivatives in hand, we investigated if the prepared compounds retained the biological activity of the parent compound and therefore conducted a series of bioassays with the MC-LR derivatives. The inhibitory effect of labelled MC-LR in comparison to unlabelled MC-LR was tested against protein phosphatase 2A. Initial tests showed that the fluorophores themselves were not interfering with the absorbance and the emission of the enzyme assay substrate, which allowed the use of compound **1** and **2** in the inhibition assays as test substrates (**Figure 1**). These derivatives were chosen to investigate if a negative charge in the compounds has a different influence on the inhibitory activity. For this purpose, the PP2A enzyme was incubated with different MCs derivatives in different concentrations (0.004 nM up to 40 nM) and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as the substrate. The activity of the non-inhibited PP2A enzyme reaction is associated with the measured fluorescence of released 6,8-difluoro-4-methylumbelliferone. The data in Figure 1 show that both MC-LR-(5,6-FAM) and MC-LR-(Alexa-430) inhibit PP2A with roughly equal IC_{50} values of 0.37 nM and 0.61 nM, respectively. These measured values for the derivatives are one order of magnitude weaker in comparison with MC-LR, which displays an IC_{50} value of 0.05 nM.[33]

Figure 2. Cell viability in Huh7 cells with MC-LR-(Alexa-430), EC_{50} = 16.3 µM

To examine the cellular uptake and the toxicity of the MC-LR derivatives, MTT cytotoxicity assays for cell viability were performed. Huh7 cells were incubated with different concentrations of compound **2** for 24 h. Additionally, fluorescent imaging of the cells was performed after 4 h to visualize the uptake of the labelled toxin. In comparison to MC-LR (4.4 µM[15]) an EC_{50} value of 16.3 µM for compound **2** could be determined, although no complete cytotoxicity could be obtained (**Figure 2**).

Figure 3. Fluorescent imaging of Huh 7 liver cells **A:** Fluorescent image of cells exposed to 100 µM MC-LR-(Alexa-430) **B:** Phase Contrast of cells exposed to 100 µM MC-LR-(Alexa-430) **C:** Fluorescent image of cells exposed to 12.5 µM MC-LR-(Alexa-430) **D:** Phase Contrast of cells exposed to 12.5 µM MC-LR-(Alexa-430) **E:** Fluorescent image at 100 µM with hydrolysed Alexa-430 **F:** Phase Contrast of cells exposed to 100 µM hydrolysed Alexa-430

In addition, visualization of the treated cells revealed that the compound could be detected in the cells (**Figure 3**). Uptake studies with the fluorophores alone did not reveal cellular uptake. Therefore, compound **2** demonstrated efficient cell uptake with retained cytotoxicity.

We next analysed the toxicity of the modified compounds **1** and **2** against aquatic organisms. The freshwater crustacean *Thamnocephalus platyurus* was chosen for the acute toxicity test, as this animal is a widely used for ecotoxicological studies.[37] Both compounds were administered over 24 h, and LC_{50} values of 5.7 µM for **1** and 15.7 µM for **2** could be determined. Again, these values are comparable to the parent toxin MC-LR (10.8 µM)[33] (**Figure 4**).

Figure 4. Acute toxicity assay **A:** with MC-LR-(5,6-FAM), LC_{50} = 5.7 µM **B:** with MC-LR-(Alexa-430), LC_{50} = 15.7

Fluorescent imaging of animals treated with MC-LR-(Alexa-430) was revealed that the compound was only accumulated in the digestive track (**Figure 5**).[38]

Figure 5. Fluorescent imaging of MC-LR-(Alexa-430) treated *Thamnocephalus platyurus* **A:** Fluorescent image at 100 µM with MC-LR-(Alexa-430) **B:** Phase Contrast at 100 µM with MC-LR-(Alexa-430)

Conclusion

In conclusion, we have successfully developed a mild and operatively simple derivatization method for MC-LR in moderate to good yields. This method allows for the modification of the toxin with a variety of biologically important tags, such as fluorescent labels, diazirine and biotin tags. Furthermore, we demonstrated that the MC-LR derivative remains active towards one of the primary target, the enzyme protein phosphatase 2A (IC_{50} value of 0.37 nM) and is active towards aquatic organisms (LC_{50} value of 5.7 µM). In addition, the tagged MC-LR successfully undergoes cellular uptake by Huh 7 cells and retains toxicity (EC_{50} value of 16.3 versus 4.3 µM for MC-LR). Additional ongoing studies aim to investigate the uptake of the labelled MC-LR derivatives in various animals using these fluorescent probes.

Experimental Section

General

The following reagents were purchased and used as received: NHS-diazirine (Thermo Scientific), all reactive succinimidyl ester fluorophores (Invitrogen), Seaplaque agarose (Lonza), all further chemicals were purchased from Sigma-Aldrich Co. and were of analytical grade. Microcystin-LR was purchased from Enzo Life Sciences. *Thamnocephalus platyurus* for the acute toxicity assay was purchased from MicroBioTests Inc., Belgium. The protein phosphatase 2A enzyme was purchases from Promega, USA. 6,8-difluoro-4-methylumbelliferyl phosphate was received from Molecular Probes, Leiden, The Netherlands. HuH7 cells were originally provided by M. Heim, University Hospital Basel, Switzerland.

For measuring the fluorescence in the protease inhibition assays a fluorescence microplate reader (Spectra MAX gemini XS; Molecular Devices Cooperation, Sunnyvale, California, USA) was used set at λ_{em} = 365 nm, λ_{ex} = 444 nm. All reactions were carried out in oven-dried glassware under an atmosphere of argon. HPLC purifications of the functionalized MC-LR was obtained on a Dionex P-680 HPLC System with a Phenomenex Gemini C18 5 µ (250 mm x 4.6 mm) column or a Phenomenex Gemini C18 5 µ (150 mm x 4.6 mm) column using a linear gradient: 5 % to 100 % CH_3CN in 0.1 % Formic acid/H_2O over 40 minutes at a flow rate of 1 mL/min. For removal of the formic acid a final purification step was performed on a C_{18} SPE cartridge (500 mg, Supelco) starting with a first washing step with water to remove the acid and a final elution step with 80 % acetonitrile.

The yield of modified MC-LR was determined using a NanoDrop 2000 Spectrophotometer (Thermo scientific). UV-Vis data was recorded on an Agilent 8453 spectrophotometer, the fluorescence was measured on a Shimadzu 5301PC spectrofluorophotometer and the quantum yield was determined on a Hamamatsu absolute PL quantum yield spectrometer C11347 Quantaurus_QY. ^{1}H-NMR spectra was recorded on a Bruker Avance III Ultrashield 600MHz with a 5 mm BBFO+ plus SP probe or a Bruker Avance III Ascend 700MHz with a 5 mm TCI (H-C/N-D) cryo probe spectrometers at room temperature. HRMS spectra were recorded on a Bruker maXis 4G instrument or measured by the mass spectrometric service of University of Bern on a *Sciex QSTAR Pulsar mass spectrometer*. HRMS spectra were obtained on a Bruker maXis 4G with ESI in positive or negative mode. MS-MS experiments were obtained on a Bruker Esquire 3000 with ESI in positive mode.

Cell culturing, extraction and isolation

M. aeruginosa UV-006 was cultivated in 500 mL Falcon tubes in mineral medium (200 mL) at 25 °C with 12h light and night cycle.[39] The resulting biomass was harvested every three months with a 6K15 centrifuge (Sigma), freezed and stored at -20 °C. To obtain pure MC-LR the cyanobacteria were suspended in 60 % acetonitrile (20 mL) per 1 g biomass and sonicated for 10 min (Branson 2510). The resulting homogenous mixture was centrifuged for 15 min at 25000 x g and the supernatants were combined and evaporated in a rotary evaporator (Büchi, Switzerland). The residue was dissolved in 60 % acetonitrile and prepurified on a C_{18} SPE cartridge (10 g, Supelco). The crude mixture was eluted with 80 % acetonitrile (500 mg biomass afforded 15 mg crude extract), concentrated and applied on a Dionex P-680 HPLC System with a Phenomenex Gemini-NX C18 5µ (75 mm x 21.2 mm) column using a linear gradient: 5 % to 100 % CH_3CN in 0.1 % Formic acid/H_2O over 40 minutes at a flow rate of 5 mL/min for further purification. For removal of the formic acid a final purification step was performed on a C_{18} SPE cartridge (500 mg, Supelco) starting with a first washing step with water to remove the acid and a final elution step with 80 % acetonitrile. After removal of all volatiles and lyophilisation pure MC-LR (600 µg from 15 mg crude extract) was afforded as a white solid. HPLC: t_R = 18.1 min; MS (ESI): *m/z* = 995.9 $[M+H]^+$

Synthesis of the modified MC-LR derivatives

General procedure for the functionalization MC-LR

To a solution of MC-LR (1 eq.) in DMF (50 µL) were added Barton's base (10 eq.) and the activated ester (1.5,1.1 or 4 eq.) in DMF (20 µL). The resulting mixture was stirred at 40 °C for 8h. All volatiles were removed under reduced pressure and the residue was purified by HPLC.

MC-LR-(5,6-FAM), (1):

According to the general procedure, compound **1** was synthesized starting from MC-LR (0.40 mg, 0.40 µmol). Barton's base (0.70 mg, 4.09 µmol) and 5(6)-carboxyfluorescein *N*-succinimidyl ester (0.22 mg, 0.46 µmol) were used. Direct purification by HPLC afforded **1** (423 µg, 0.31 µmol, 84 %) as a red solid. HPLC: t_R = 23.6 min; HRMS-ESI: calcd. for $C_{70}H_{85}N_{10}O_{18}^+$ $[M+H]^+$: 1353.6038; found: 1353.6087.

MC-LR-(Alexa-430), (2):

According to the general procedure compound **2** was synthesized starting from MC-LR (1.00 mg, 1.00 µmol). Barton's base (1.10 mg, 6.43 µmol) and Alexa 430 succinimidyl ester (0.78 mg, 1.11 µmol) were used. Direct purification by HPLC afforded **2** (1.36 mg, 0.92 µmol, 74 %) as a red solid. HPLC: t_R = 33.4 min; HRMS-ESI: calcd. for $C_{71}H_{95}N_{11}O_{18}F_3S^-$ $[M-H]^-$: 1478.6524; found: 1478.6527.

MC-LR-(Alexa-488), (3):

According to the general procedure compound **3** was synthesized starting from MC-LR (0.20 mg, 0.20 µmol). Barton's base (0.34 mg, 2.0 µmol) and Alexa 488 5-sulfodichlorphenol ester (0.25 mg, 0.30 µmol) were used. Direct purification by HPLC afforded **3** (0.17 mg, 0.11 µmol, 55 %) as a red solid. HPLC: t_R = 10.9 min; HRMS-ESI: calcd. for $C_{70}H_{84}N_{12}O_{22}S_2^{2-}$ $[M-H-H]^{2-}$: 754.2638; found: 754.2625.

MC-LR-(Texas-Red), (4):

According to the general procedure compound **4** was synthesized starting from MC-LR (0.50 mg, 0.50 µmol). Barton's base (0.86 mg, 5.02 µmol) and Texas Red succinimidyl ester (0.50 mg, 0.61 µmol) were used. Direct purification by HPLC afforded **4** (650 µg, 0.38 µmol, 76 %) as a red solid. HPLC: t_{R1} = 23.6 min; t_{R2} = 25.6 min; HRMS-ESI: calcd. for $C_{86}H_{114}N_{13}O_{19}S_2Na^{2+}$ $[M+H+Na]^{2+}$: 859.8841; found: 859.8852.

Phosphatase inhibition assays

The enzyme mixture was prepared as followed: PP2A enzyme (2 µL) was dissolved in EGTA (650 µL, 1 mM), DTT (50 µL, 10 mM in 5 mM sodium acetate pH = 5.2), $MnCl_2$ (200 µL, 6 mM) and BSA (100 µL, 5 mg/mL). The reaction buffer was prepared out of $MnCl_2$ (290 µL, 6 mM), $MgCl_2$ (290 µL, 0.8 M) and BSA (290 µL, 5 mg/mL). In a 200 µL well plate 25 µL of the enzyme mixture was used. To achieve a final concentration of 25 mU PP2A (200 µL), Tris-HCl (30 µL, 50 mM, pH = 7.0) and the reaction buffer (15 µL) were added. The mixture was incubated for 10 minutes at 37 °C. Afterwards 10 µL of **1** and **2** were added in various concentrations (0.004 nM up to 40 nM). Only the fluorophores were used as control experiment to test possible interference with the experimental setup. Subsequent incubation at 37 °C for 10 minutes was followed. To start the measurement 6,8-difluoro-4-methylumbelliferyl phosphate (120 µL, 10 mM dissolved in 50 mM Tris-HCl pH = 7.0) solution was added. After a final incubation over 5 minutes at 37 °C the fluorescence measurement was started by taking a data point every 30 seconds for 45 minutes. To obtain the activity of the phosphatase, linear regression was used using Graph Pad Prism 4.1 for Windows.

MTT assay for cytotoxicity

Human hepatoma cells (Huh7) cells were plated after growing in DMEM containing GlutaMAX (LuBioSciene) with 10 % FBS in a humidified incubator (5 % CO_2, 37 °C), in a 96-well plate at a density of 50000 cells per condition in 50 µL fractions. After 24 h of incubation, a series of diluted solution of compound **2** in medium without FBS was added to the cells. After additional 24 h, the mixture was replaced with fresh medium (200 µL). Pictures of the uptake into the cells were taken with an Olympus CKX41 biological microscope with a 10x objective. Analysis of the pictures were performed with Cell B Version 3.2. Afterwards MTT (20 µL, 5 mg/mL) was added, followed by an incubation over 2 h at 37 °C. Subsequently, the solution was removed and DMSO (200 µL) was added and mixture was shaken for 15 min. Soerensen buffer (25 µL) was added and absorbance was measured at 570 nm on a plate reader.

Acute toxicity assay

To determine the acute toxicity of the modified MC-LR, compound **1** and **2** was utilized in an acute toxicity assay with *Thamnocephalus platyurus* in a multiwell plate using instar II-III larvae hatched from cysts during 24 hours. Compounds **1** and **2** were tested in various concentrations (0.5 to 100 µM) using 30 animals per condition. The LC_{50} value was calculated using the nonlinear regression Graph Pad Prism 4.1 for Windows was used.

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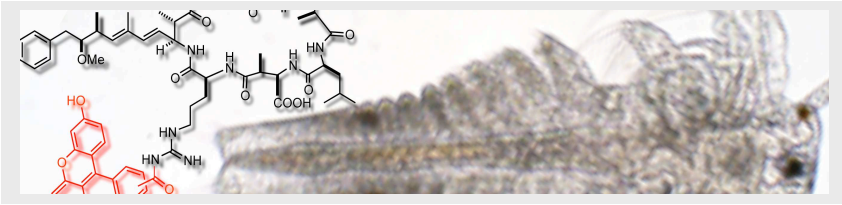

A series of fluorescently labelled microcystin derivatives has been prepared via direct arginine labelling in native peptides. A series of toxicity, environmental fate studies in crustaceans, as well as enzyme and cellular assays have been carried out and demonstrated the usefulness of these probes in complex biological systems.

Verena Grundler, Susanne Faltermann, Karl Fent and Karl Gademann[*]*

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Preparation of Fluorescent Microcystin Derivatives via Direct Arginine Labelling and their Biological Evaluation